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Mechanisms Underlying Regulation of Local Immune Responses in the Uterus during Early Gestation of Eutherian Mammals. III. Possible Functional Differentiation of Macrophages Cultured Together with Blastocyst *in vitro*, with Special Reference to the Cellular Shape and Production of Leukotriene C_4

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ABSTRACT—In the blastocyst-macrophage co-culture of the mouse, we found two major groups of macrophages which were different in the morphology, i.e., the rounded cells, and the elongated cells. The macrophages in direct contact with the embryonic cells, regardless of whether they were trophoblast or ICM cells, assumed invariably rounded cellular shape. Colchicine, cytochalasin B and D induced strong rounding of the macrophage. The rate of synthesis of leukotriene C_4 in those rounded macrophages remained at a low level of the unstimulated cells. Therefore, it was tentatively proposed that in the blastocyst-macrophage co-culture, the rate of production of LTC_4 in the rounded macrophages, might remain at a low level, while in the elongated ones the rate might be enhanced [C. Tachi and U. Zor, Zool. Sci., in press]. On the other hand, tuftsin, a naturally occurring tetrapeptide known to augment phagocytosis as well as capability of antigen presentation in macrophages, raised slightly the rate of LTC_4 synthesis around the concentration of 60 nM or above. Recently Gupta presented evidence indicating that LTC_4 mediates the initial estrogen-dependent phase of endometrial modification during nidation in mice. While it is strongly suspected that the major source of LTC_4 produced during that period, might be one of the functionally differentiated groups of macrophages in the endometrium, further work is needed to corroborate the view.

INTRODUCTION

We proposed [1–3], on the basis of the observations made in the rat and the mouse, that macrophages are probably involved in the local immune responses elicited in the endometrium by implanting blastocysts during early gestation of eutherian mammals. We suggested [1–3], furthermore, that the decidua might function to limit the access of macrophages to the embryonic antigens, regulating the afferent flow of immunological information to the maternal immune system during the initial phase of the recognition of the conceptuses. However, the precise role played by the endometrial macrophages during implantation and/or nidation that is one of most critical episodes in the true viviparity of mammals, remains yet to be clarified.

As an approach to the problem, we analyzed, at ultrastructural levels, the mode of cell-to-cell interactions between the blastocysts and macrophages cultured together *in vitro* [4]. During the course of experiments, it came to our notice that the macrophages in the co-culture assumed, as will be described in this report in detail, mainly two distinctly different cellular shapes, i.e., they were either rounded or elongated.

Macrophages have been known to change their shape according to their functional states or to the

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changes in their environmental conditions [5–10]. In turn, it is possible to bring about functional transformation of the macrophages cultured *in vitro* by exposing them to compounds which affect the cytoskeletal organization of the cells. Thus, colchicine, cytochalasin B and vinblastin stimulate the release of neutral peptidases, including elastase [11, 12], collagenase [11], gelatinase [11], azocaseinase [11] from the cultured peritoneal [11] as well as alveolar [12] macrophages of the mouse. Colchicine stimulates also the rate of production of interleukin-1 [13], in the irradiated peritoneal macrophages of the rat. Calcium ionophore A23187 added to the cultured peritoneal macrophages of the mouse [Tachi and Zor, Zool. Sci., in press] induced at alkaline pH's of the medium, strong elongation of the cellular shape, accompanied by the highly accerelated rate of leukotriene C_4 (LTC_4 in the following) production; stimulation of the Ca^{++}-dependent synthesis of LTC_4 [14] by A23187 in macrophages has been reported earlier in the literature [15–17].

Leukotrienes, as termed by Samuelsson *et al.* [18], are a family of metabolites of arachidonic acid, and produced via 5-lipoxygenase pathway. Leukotriene C_4 is the first peptidolipid to be synthesized in the family and held responsible for mediating inflammation, asthma and many other functions of leukocytes and/or macrophages [for review see, 19–25] which are the major source of LTC_4 in the mammalian body. Recently, in 1989, Gupta *et al.* [26] proposed that LTC_4 might be one of the mediators of the endometrial changes elicited during the estrogen-dependent early phase of implantation in the mouse, although the exact source of LTC_4 produced during the period is yet to be determined.

In order to examine the possibility that the morphological difference of the macrophages in the blastocyst-macrophage co-culture might reflect the underlying functional difference which, in turn, might be correlated with the rate of LTC_4 synthesis in the phagocytes, we analyzed the distribution of macrophages according to their morphology in the co-culture, and at the same time, we assayed the rate of LTC_4 production in the macrophages under the influence of the compounds which affect the cytoskeletal organization of the cells. It was hoped that such analyses will provide us clues to understand the mechanisms underlying the blastocyst-endometrial interactions during implantation and/or nidation in eutherian mammals.

Part of the results has been presented in abstract form [27–29].

MATERIALS AND METHODS

Animals

Specific pathogen free (SPF) mice of BALB/c (H-2^d) and C3H/HeJ (H-2^k) strains were used throughout the experiments. They were purchased from a local dealer (Nippon Clea & Co., Ltd., Tokyo, Japan), and had been kept in the animal room of our laboratory under regulated temperature (25°C) and illumination cycles (12 hr dark and 12 hr light per day), until they were used for the experiments. Female BALB/c mice were mated on the day of proestrus with fertile BALB/c males, and if the vaginal plugs were found next morning, the day was counted as Day 1 of pregnancy.

Drugs and reagents

Unlabelled LTC_4 was purchased from Wako Pure Chemical Industries & Co., Ltd. (Osaka, Japan). Calcium ionophore A23187, cytochalasin B and D were obtained from Sigma Chemical & Co., Ltd. (St. Louis, Mo, USA): each of the three compounds was dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries) at a concentration of 500 μg/ml. Colchicine (Merck, Darmstadt, West Germany) and Tuftsin (Wako Pure Chemical Industries), a polypeptide known to be an activator of macrophages, were dissolved in glass distilled water at a concentration of 500 μg/ml. Sterilized Ficoll-Hypaque solution ($d=1.090\pm0.001$) was purchased from Otsuka Assay Loboratories (Tokushima-shi, Japan).

Radioactive LTC_4

Aqueous solution of tritium-labeled leukotriene C_4 (specific activity, 39 Ci/mMol, Amersham Japan & Co., Ltd., Tokyo, Japan), as supplied by its manufacturer, was diluted with 50% ethanol to give a final concentration of 1.0 μCi/ml.

Macrophages

The macrophages were collected from peritoneal exudate of C3H/HeJ mice by injecting 60 units of heparin dissolved in Earl's minimum essential medium (MEM) at a concentration of 30 units/ml under ether anesthesia. After 3–5 min, the animals were killed by cervical dislocation and the peritoneal fluid was gently aspirated into a glass syringe.

For the co-culture experiments, the procedures previously described were essentially followed [4]. The macrophages were collected by centrifugation at approximately 1000 rpm for 5 min, and washed three times with phosphate-buffered saline (PBS) and added to the culture medium which is known to favor the trophoblast spreading [30].

For the assay of LTC_4, and the examination of the effects of various compounds which affect the cytoskeletal organization of the cells, highly purified peritoneal macrophages were prepared as described previously [28, C. Tachi and U. Zor, Zool. Sci., in press].

Blastocysts

Blastocysts were collected from BALB/c mice as described before [4] by flushing the uterine lumen with the standard egg culture medium (SECM) [31] on Day 4 of pregnancy. The collected embryos were washed twice in SECM and introduced into the culture of macrophages.

Co-culture of blastocysts and macrophages

The zona-encased blastocysts (BALB/c) collected in the afternoon of Day 4 from the uterine lumen were introduced to the culture of macrophages (C3H/HeJ); usually 5 blastocysts were added to a single Falcon dish (diameter of the dish, 34.5 mm) which contained macrophages allogeneic to the embryos at an approximate concentration of $1\text{–}3\times10^6$ cells per dish. The co-cultures were incubated at 37°C under the atmosphere of 5% CO_2 and 95% air.

Determination of the rate of LTC_4 production

The rate of LTC_4 production was determined, as will be described elsewhere [C. Tachi and U. Zor, Zool. Sci., in press], by assaying the total amount of the cysteinyl leukotrienes released into the medium from the macrophages during 1.5–2.0 hr of the culture period. The radioimmunoassay of the cysteinyl leukotrienes were done essentially following the procedures described by Danzlinger *et al.* [32] with minor modifications; the monoclonal antibodies against cysteinyl leukotrienes were generous gift of Dr F. Kohen, Department of Hormone Research, Weizmann Institute of Science, Rehovot, Israel. The antibodies reacted with LTC_4 (100%), LTD_4 (105%), and LTE_4 (77%) at a 50% saturation level of binding [F. Kohen, personal communication].

One tenth ml of the sample solution to be assayed was mixed with an equal amount of the antibody solution and incubated at 0°C for 30 min. Then, 0.1 ml of $^3H\text{-}LTC_4$ solution containing 7 nCi of the isotope, was added to the mixture, and had been stood at 4°C overnight. Free LTC_4 was removed by adding 0.2 ml of dextran-charcoal, and by centrifugation at 15,000 rpm for 3 min. The radioactivity was measured by liquid scintillation counting.

Differential counting of macrophages according to their cell shape

The photomicrographs of the co-cultures were taken using a phase contrast microscope (Model CK, Olympus & Co., Tokyo, Japan), approximately 72 hr after the initiation of the culture. For histological examination, the cells were fixed with 3.5% formaldehyde solution and stained with Giemsa's. The number of the cells of the elongated, or the rounded cellular shape were differentially counted on the photomicrographs, by using a microcomputer-based graphic analysis system (TACSYS/G) previously described by C. Tachi [2].

RESULTS

1. *Cellular shape of the macrophages cultured together with blastocysts in vitro*

Microscopic appearance of macrophages adhered to the zona pellucida or the blastocysts following the co-culture of the phagocytes with the zona-encased embryos, is shown in Figure 1A.

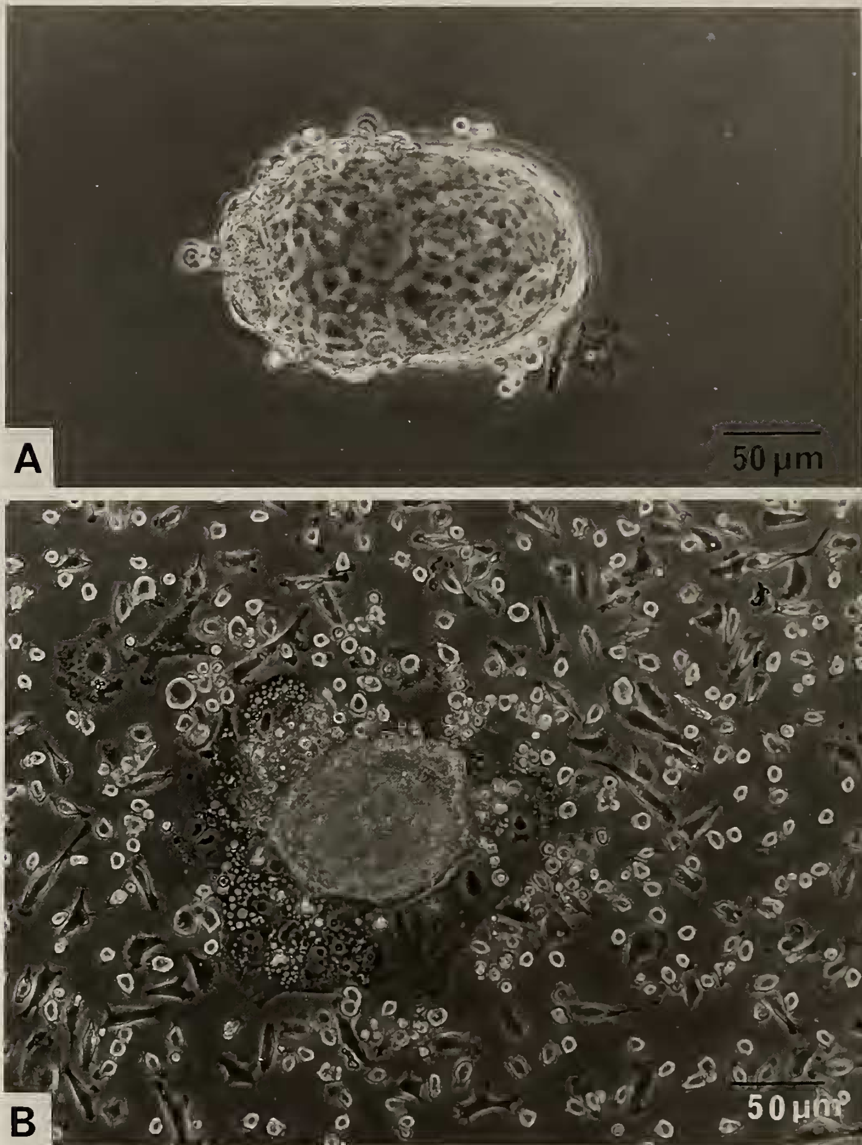

FIG. 1. Photomicrographs of blastocyst-macrophage co-culture. A) The blastocyst (BALB/c) is encased in zona pellucida onto which numerous macrophages (C3H/HeJ) are firmly adhered. Approximately 6 hr after the initiation of co-culture. B) The blastocyst is undergoing trophoblast spreading. Approximately 72 hr after the initiation of co-culture.

The macrophages which were tightly bound to the zona, assumed strongly rounded cellular shape without exception; none of the zona-bound phagocytes observed was of elongated morphology.

In Figure 1B, representative photomicrograph of the blastocyst-macrophage co-cultures approximately 48 hr after the initiation, is presented. The blastocyst was undergoing trophoblast spreading and numerous macrophages were found in contact with the periphery of the trophoblast cell layer. Some of the macrophages were located within the embryos (Fig. 1B).

In the co-culture, two groups of macrophages of distinctly different morphology were discernible,

i.e., those which were rounded and the others which were either elongated or spread (Fig. 1B). The macrophages with spread morphology, however, were only rarely observed under the conditions we employed. We counted the number of macrophages according to their cellular shape, and the distance from the periphery of the trophoblast spread. The results are presented in Table 1. Almost all the macrophages in contact with the spreading trophoblast cells, were rounded (Table 1); no elongated macrophages were found inside the blastocysts. While the macrophages lying in close vicinity of the embryos (less than 20 μm from the edge of the trophoblast spread) tended to assume the rounded morphology, the difference in the relative frequencies of the rounded cells, from those in other areas was not statistically significant.

2. *Drug-induced rounding of macrophages and production of LTC_4* in vitro

In order to understand the mechanisms underlying the rounding of the macrophages, induced when they are in contact with the embryonic cells, we tested the effect of various compounds which are known to affect the cytoskeletal organization of the cells. Furthermore, the rate of production of LTC_4 in the phagocytes under the influence of those compounds was examined.

In Figure 2A-D, the photomicrographs show the effects of colchicine, cytochalashin B and D upon the cellular shape of highly purified peritoneal macrophages of the mouse. All the three compounds induced strong rounding of the cells at the concentrations indicated in the legends to the figures. Tuftsin, an activator of macrophages, had little effects upon the morphology of the cells (Fig. 2E).

In Figure 3A, the relative contents of the macrophages of the rounded and the elongated morphology in the culture, are presented according to the concentrations of colchicine added. At the concentrations above 5 μg/ml, all the macrophages were seen rounded. The rates of LTC_4 production in the same cell populations are shown in Figure 3B. Colchicine affected little the rates which remained unchanged after the addition of the compound to the cells, and stayed at a low level throughout the range of the colchicine concentrations examined.

Cytochalasins, both B and D, at sufficiently high concentrations, resulted in the complete rounding of the phagocytes in the culture (Fig. 4A, 5A). The rate of production of LTC_4, however, remained at the control level throughout the range of concentrations of the compounds examined (4B, 5B).

TABLE 1. Distribution of macrophages with either elongated or rounded cellular shape in blastocyst-macrophage co-culture of allongeneic combinations[1]

Location of Macrophages	No. of Experiments	Rounded Macrophages		Elongated Macrophages	
		No. of Cells	Relative Frequency	No. of Cells	Relative Frequency
		cells/embryo[2]	%	cells/embryo	%
On the Zona Pellucida	6	54.0±13.7	100.0	0.0± 0.0	0.0
		cells/embryo	%	cells/embryo	%
Within 20 μm from Edge of Trophoblast Spread	12	39.5± 9.6	75.8	12.6± 3.9	24.2
		cells/0.1 mm^2	%	cells/0.1 mm^2	%
Areas Surrounding the Embryo (Further than 20 μm from the Edge)	12	78.8±25.0	61.5	49.0±11.2	38.5

[1] Blastocysts were obtained from BALB/c mice, and macrophages were collected from C3H/HeJ mice.
[2] Only the number of macrophages on a hemisphere of the zona facing toward the observed through the lenses, was actually counted. The values obtained for the hemisphere were multiplied by a factor of 2, and presented as number of cells per embryo.

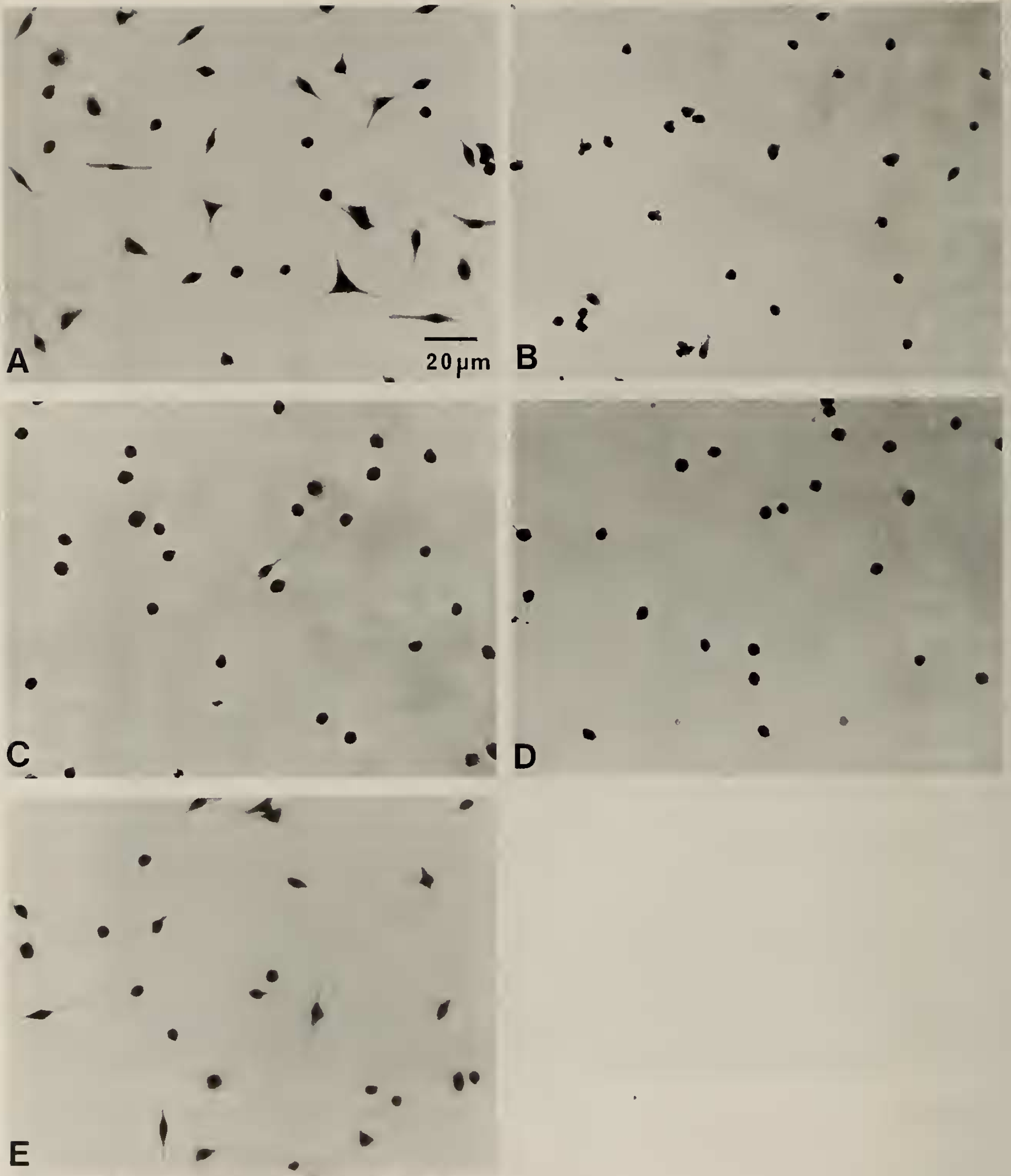

FIG. 2. Photomicrographs showing the effects of druge which affect the cytoskeletal organization of cells, upon the morphology of macrophages *in vitro*. A) Control with no additions; B) colchicine (500 μng/ml); C) cytochalasin B (500 ng/ml); D) cytochalasin D (500 ng/ml); E) Tuftsin (60 nM/ml).

3. *Effects of tuftsin upon the cellular morphology and the rate of production of LTC_4*

Tuftsin, a polypeptide known to be a stimulator of macrophage activities [33–41], did not induce noticeable changes in the morphology of these cells (Fig. 6A). It did not cause significant changes in the rate of LTC_4 production, except at 60 nM where slight but significant elevation in the rate was observed (Fig. 6B).

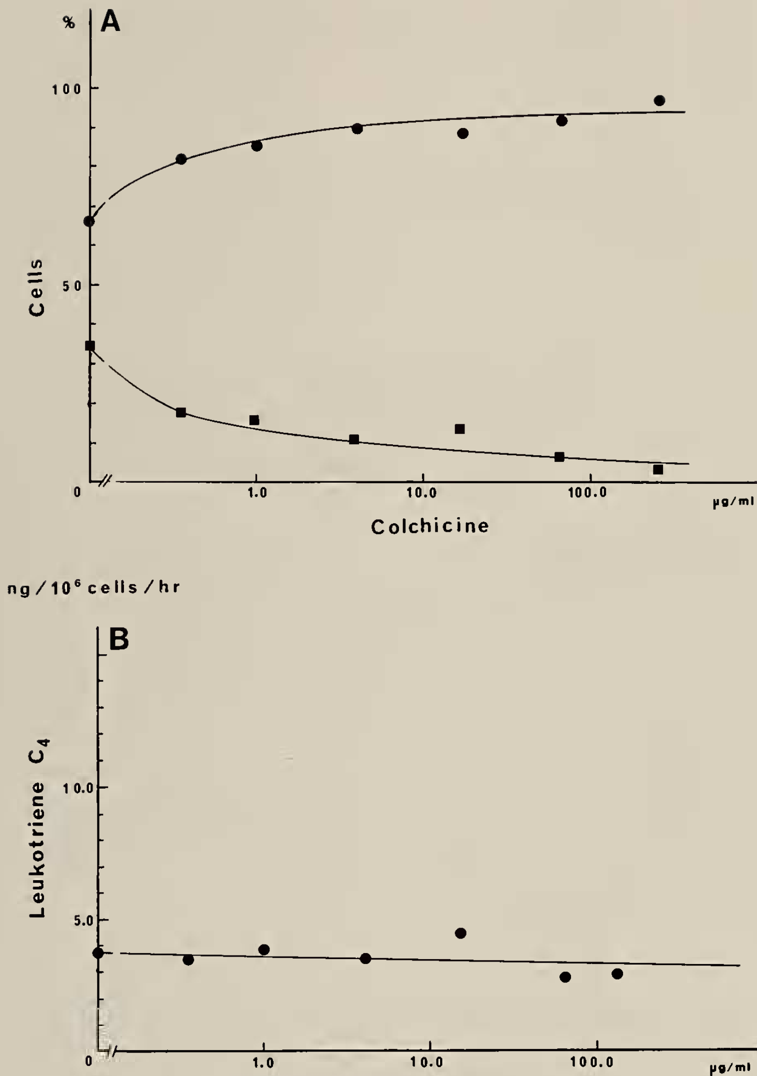

FIG. 3. Effects of colchine upon the macrophages cultured *in vitro*. A) Morphology. ●, rounded cells; ■, elongated cells. B) The rate of production of leukotriene C_4 assayed as the total amount of cysteinyl leukotrienes released into the medium (see Materials and Methods).

DISCUSSION

Present report demonstrated that in the blastocyst-macrophage co-culture of the mouse, two major morphologically distinct groups of macrophages, i.e., those which were rounded, and the others which were elongated, are present, and that the phagocytes in direct contact with the embryonic cells, regardless of whether they were trophoblast or ICM cells, assumed invariably rounded cellular shape. Evidence was presented, furthermore, indicating that the rate of synthesis of cysteinyl leukotrienes in the rounded macrophages is probably low, and remains at the level of unstimulated macrophages.

Fauve *et al.* [42], as a part of their studies upon

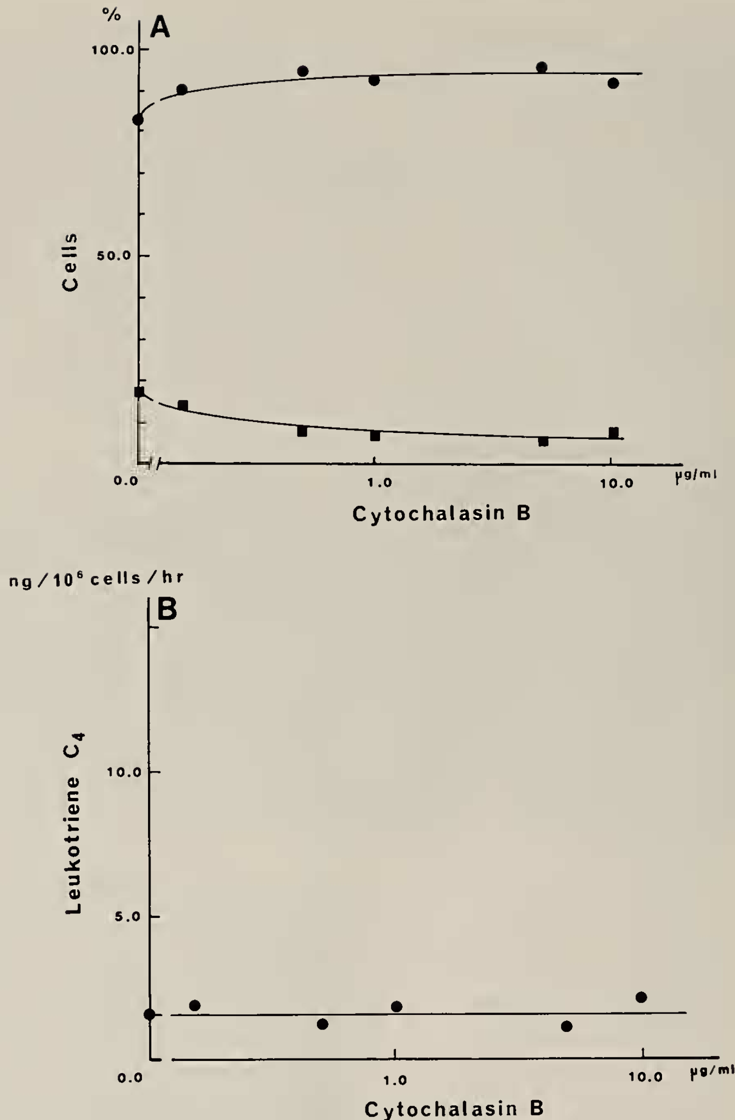

FIG. 4. Effects of cytochalasin B upon the macrophages cultured *in vitro*. A) Morphology. ●, rounded cells; ■, elongated cells. B) The rate of production of leukotriene C_4 assayed as the total amount of cysteinyl leukotrienes released into the medium (see Materials and Methods).

the anti-inflammatory effects of murine malignant teratocarcinoma cells, examined the interactions between the trophoblast cells and the macrophages cultured together *in vitro*. They noted [42] that in the vicinity of the trophoblast cells, the macrophages were unable to spread but became necrotic. They suggested that the trophoblast cells, like the teratocarcinoma cells, might escape the immunological surveillance of the host by exerting a direct cytotoxic effect on macrophages, and by releasing a hypothetical inhibitor of inflammatory reactions.

We could not convincingly observe, however, the evidence for explicit cytotoxic influence of the trophoblast cells upon the macrophages.

The peritoneal macrophages used by Fauve *et al.* [42] were of considerably high purity [43]. However, possibility cannot be entirely excluded that the

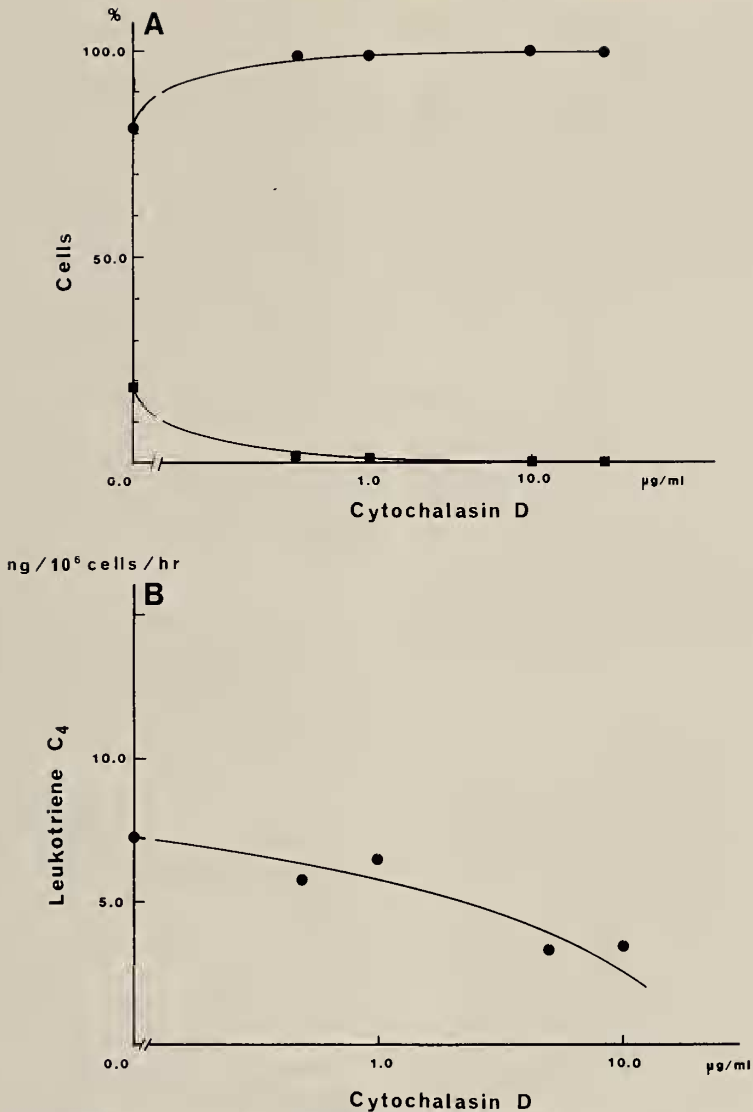

FIG. 5. Effects of cytochalasin D upon the macrophages cultured *in vitro*. A) Morphology. ●, rounded cells; ■, elongated cells. B) The rate of production of leukotriene C_4 assayed as the total amount of cysteinyl leukotrienes released into the medium (see Materials and Methods).

necrotic cells were not macrophages but lymphocytes which adhered non-specifically to the culture dishes, and contaminated the macrophage preparation.

We, too, occasionally observed in the blastocyst-macrophage co-culture, cells which resembled dead macrophages and were ingested by the trophoblast cells [4]. Although we could not definitely identify the type of the ingested cells, we considered it rather unlikely that these cells represented the macrophages actively killed and ingested by the trophoblast cells [4].

Under the experimental conditons we employed, colchicine, cytochalasin B and D induced strong rounding of the cellular shape in the macrophages.

According to the observations reported earlier by White *et al.* [12], while colchicine caused round-

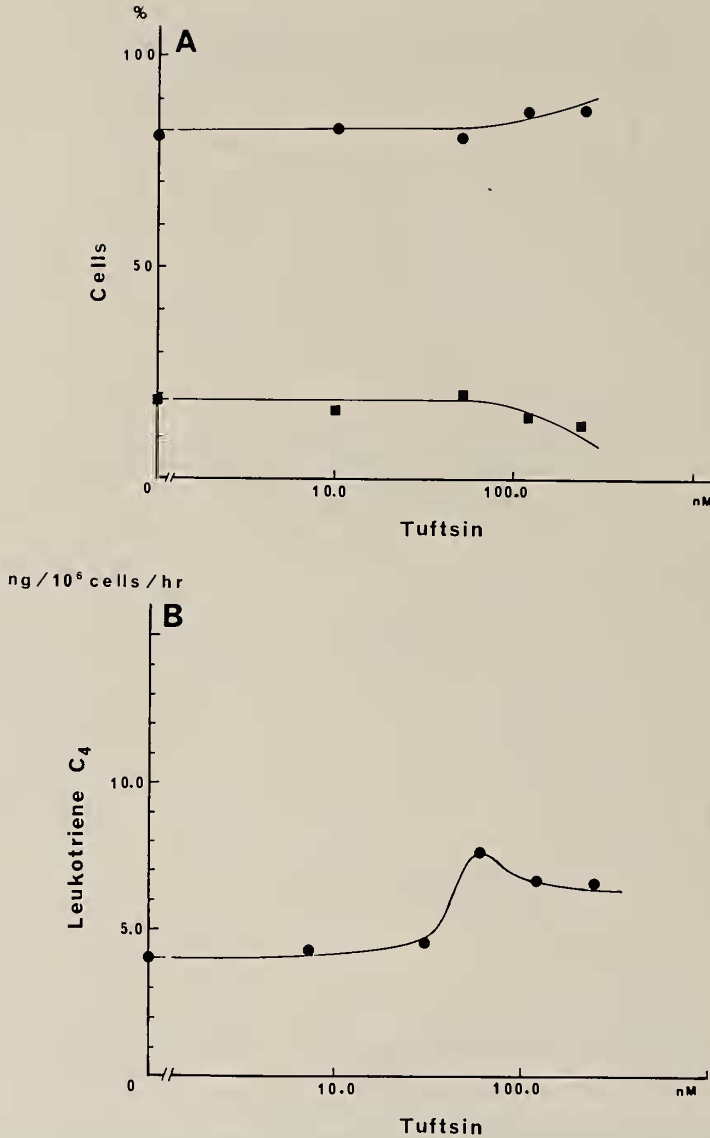

FIG. 6. Effects of tuftsin upon the macrophages cultured *in vitro*. A) Morphology. ●, rounded cells; ■, elongated cells. B) The rate of production of leukotriene C_4 assayed as the total amount of cysteinyl leukotrienes released into the medium (see Materials and Methods).

ing of the cultured macrophages, cytochalasin B had little effect upon the morphology of the phagocytes; both of the drugs were added to the cells at a concentration of 10^{-6} M. The cause for the discrepancy between our results and those described by White *et al.*, is not clear.

Macrophages are one of the major sources of leukotrienes in the mammalian body, and these metabolites of arachidonic acid are known to mediate variety of pathological conditions, including inflammation, allergy, asthma etc. (for reviews see [20–25]). While the production of LTC_4 is dependent upon the increased intracellular levels of calcium, macrophages contain a cytoskeletal protein, gelsolin, the activity of which is regulated by calcium [44–46]. Indeed, Ca ionophore added

to macrophages at alkaline pH's of the medium, induced strong elongation of the macrophages, while the rate of leukotriene C_4 production increased approximately 100-fold [C. Tachi and Zor, Zool. Sci., in press].

Since, as stated in Introduction, the disturbance of cytoskeletal organization in the phagocytes by drugs, results in the increased rate of release of various molecules of biological activity from the cells, it is pertinent to ask if the morphological changes of macrophages induced by the contact with the embryos, or by the drugs, might results in the modified rate of synthesis of leukotrienes.

Our results presently described, however, clearly demonstrated that the macrophages of the rounded morphology induced by the compounds which affect the cytoskeletal organization of the cells, were inactive with regard to the release of the cysteinyl leukotrienes.

On the basis of those findings, we would like to tentatively propose, as a working hypothesis, that in the blastocyst-macrophage co-culture, the rate of production of LTC_4 in the rounded macrophages, may remain at a low level, while in the elongated ones the rate might be enhanced [C. Tachi and U. Zor, Zool. Sci., in press].

However, the drug-induced transformations of the cellular morphology may not be immediately comparable to those caused in the co-culture, under the influence of the embryos. Therefore, it remains to be investigated, if the elongated and the rounded macrophages in the co-culture, are in fact synthesizing LTC_4 at different rates.

Tuftsin, a naturally occurring tetrapeptide derived from Fc segment of IgG [33–36], has been shown to specifically bind to macrophages [38], augment phagocytosis [35, 36, 38] and triggers the antigen-specific, macrophage-dependent education of T-cells [37]. The peptide appeared to raise slightly the rate of LTC_4 synthesis around the concentration of 60 nM or above.

Gupta *et al.* [26] proposed that LTC_4 might mediate the initial estrogen-dependent phase (phase I) of peri-nidatory changes of the endometrium in the mouse. Experimental evidence in support of the hypothesis implicating the role of prostaglandins [47–51] and/or leukotrienes [52–55] in the process of decidualization, has been reported in the literature.

While it is tempting to propose that the major source of LTC_4 might be one of the functionally differentiated groups of macrophages which abundantly emerge, as described originally by ourselves [1, 3], in the endometrium during the early phase of implantation, further analytical work on the functional as well as the morphological aspects of the blastocyst-macrophage interactions *in vitro* is necessary to corroborate the view.

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Note added in proof. The paper cited in the text as C. Tachi & U. Zor in press, has since been published as follows;
Tachi, C. and Tachi, S. (1989) Effect of calcium ionophore A23187 upon the rate of leukotriene C_4 production and the cellular morphology in highly purified mouse peritoneal macrophages cultured *in vitro*. Zool. Sci., **6**: 251–257.